Overall Kinetic Mechanism of 6-Phosphogluconate Dehydrogenase from *Candida utilis*†

Anthony J. Berdis‡ and Paul F. Cook*,§

Departments of Biochemistry and Molecular Biology and Microbiology and Immunology, Texas College of Osteopathic Medicine, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107

Received July 14, 1992; Revised Manuscript Received November 11, 1992

ABSTRACT: A complete initial velocity study of the 6-phosphogluconate dehydrogenase from *Candida utilis* at pH 7 and 25 °C in both reaction directions suggests a rapid equilibrium random kinetic mechanism with dead-end E:NADP:(ribulose 5-phosphate) and E:NADPH:(6-phosphogluconate) complexes. Like substrate–product (NADP/NADPH and 6-phosphogluconate/ribulose 5-phosphate) pairs are competitive whatever the concentration of the other substrates but noncompetitive versus the other substrates, e.g., NADPH exhibits noncompetitive inhibition versus 6-phosphogluconate. This trend also holds true for all dead-end analogs, e.g., ATP-ribose is competitive versus NADP and noncompetitive versus 6-phosphogluconate. A quantitative analysis of the kinetic inhibition constants supports the assignment of kinetic mechanism. The ratio of the maximum velocities in the oxidative decarboxylation and reductive carboxylation directions is 75.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44) catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2 with the concomitant generation of NADPH.[1] This reaction is similar in nature to that catalyzed by isocitrate dehydrogenase and malic enzyme in that all three yield a ketone, CO_2, and NAD(P)H as products. However, unlike these other enzymes, 6-phosphogluconate dehydrogenase does not require the presence of divalent metal ions for activity (Pontremoli et al., 1961; Siebert et al., 1957; Rutter & Lardy, 1958).

Numerous kinetic studies have been performed on 6-phosphogluconate dehydrogenase from various sources, but none have carried out a systematic initial velocity study to determine the kinetic mechanism. The kinetic parameters for the enzyme in the direction of oxidative decarboxylation have been determined by several groups using enzymes from rat liver (Holten et al., 1972), sheep liver (Hansen et al., 1973), and *Leuconostoc mesenteroides* (Rendina et al., 1984), while Villet and Dalziel (1972) used the sheep liver enzyme to determine the kinetic parameters in the direction of reductive carboxylation.

The kinetic mechanism for 6-phosphogluconate dehydrogenase has been proposed by several groups to be random on the basis of some product and dead-end inhibition studies Bellini et al. (1985) using human erythrocyte 6-phosphogluconate dehydrogenase and Dalziel et al. (1986) using the enzyme from sheep liver have shown that NADPH is competitive with respect to NADP and noncompetitive with respect to 6-phosphogluconate. Anderson et al. (1988), using the *Haemophilus influenza* enzyme, have shown that ATP-ribose acts as a competitive inhibitor with respect to NADP. The above patterns are consistent with several kinetic mechanisms. Product inhibition studies using ribulose 5-phosphate and/or CO_2 have not been previously reported nor have dead-end inhibition studies using an analog of 6-phosphogluconate. Product and dead-end inhibition studies in the direction of reductive carboxylation have also not been previously reported. It has been shown that the deuterium isotope effects on the V/K for both NADP and 6-phosphogluconate are finite with the enzyme from *L. mesenteroides* (Rendina et al., 1984) in agreement with the proposed random mechanism (Cook & Cleland, 1981).

In this paper, a systematic initial velocity study is carried out to determine the overall kinetic mechanism of 6-phosphogluconate dehydrogenase from *Candida utilis*. Results are consistent with a rapid equilibrium random kinetic mechanism.

MATERIALS AND METHODS

Enzyme. Cytoplasmic 6-phosphogluconate dehydrogenase from *Candida utilis* was purchased from Sigma. Enzyme was homogeneous by the criterion of SDS–polyacrylamide gel electrophoresis and had a final specific activity of 48 units/mg assayed in the direction of oxidative decarboxylation using 100 mM Hepes, pH 7, 3 mM 6-PG, and 0.4 mM NADP. The enzyme was stored at −20 °C in a storage buffer containing 20 mM Hepes, pH 7, 1 mM BME, and 20% glycerol.

Chemicals. NADP and NADPH were from Boehringer-Mannheim. 6-Phosphogluconate, ribulose 5-phosphate, glucose 6-sulfate, ribose 5-phosphate, ATP-ribose, and Hepes were from Sigma. $NaHCO_3$ was from Matheson Coleman Biochemical. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

6-Sulfogluconate and 5-phosphoribonate were synthesized by bromine oxidation of glucose 6-sulfate and ribose 5-phosphate by an adaptation of the method by Horecker (1957). Briefly, between 100 and 500 mg of glucose 6-sulfate or ribose 5-phosphate are solubilized in 20 mL of H_2O and 0.4 mL of concentrated HCl followed by the addition of 8 mL of 0.57 M Na_2SO_4. The pH is then adjusted to 5.4 by the addition of 4 N NaOH, and a molar excess of Br_2 is added. The pH is maintained between 4.8 and 6.2 for at least 20 min by the addition of 4 N NaOH. The resultant solution is then

† This work was supported by grants to P.F.C. from the NIH (GM 36799) and the Robert A. Welch Foundation (B-1031).

* Author to whom correspondence should be addressed.

‡ Department of Biochemistry and Molecular Biology.

§ Department of Microbiology and Immunology.

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; BME, β-mercaptoethanol; NADP, nicotinamide adenine dinucleotide 2′-phosphate (the plus sign is omitted for convenience); NADPH, reduced NADP; 6-PG, 6-phosphogluconate; 6-PGDH, 6-phosphogluconate dehydrogenase; Ru 5-P, ribulose 5-phosphate; 6-SG, 6-sulphogluconate; 5-PRib, 5-phosphoribonate; ATP-ribose, 2′-monophosphoadenosine 5′-diphosphoribose.

0006-2960/93/0432-2036$04.00/0

separated with a constant flow of N_2 for at least 1 h to remove any remaining Br_2. The mixture is then applied to a Bio-Rad econocolumn containing 3 mL of boronate resin and then washed with 100 mM ammonium formate, pH 9. The 6-SG or 5-PRib is then eluted from the column using five volumes of 100 mM ammonium formate, pH 5. The resulting 6-SG or 5-PRib is then concentrated by rotary evaporation, the pH adjusted to 7, and the material used without further treatment.

Initial Velocity Studies. All assays were carried out using a Gilford 250 spectrophotometer equipped with a strip-chart recorder. The appearance of NADPH was measured at 340 nm in the direction of oxidative decarboxylation, while the disappearance of NADPH was monitored in the direction of reductive carboxylation. The temperature was maintained at 25 °C using a circulating water bath to heat the thermospacers of the cell compartment. Reaction cuvettes were 1 cm in path length and 1 mL in volume. All cuvettes were incubated for at least 10 min in a water bath prior to initiation of reaction. A typical assay in the direction of oxidative decarboxylation contained 100 mM Hepes, pH 7, 200 μM NADP, 2 mM 6-PG, and 1.4 μg of enzyme. In the direction of reductive carboxylation, a typical assay contained 100 mM Hepes, pH 7, 10 mM Ru 5-P, 200 μM NADPH, 20 mM CO_2, and 110 μg of enzyme. The reactant, CO_2, was added as bicarbonate with CO_2 concentration calculated according to the Henderson–Hasselbach equation using a pK of 6.4. Assays were carried out with capped cuvettes. To be sure that CO_2 was not being rapidly lost under these conditions, the initial velocity was measured as a function of time for identically prepared cuvettes with a nonsaturating CO_2 concentration. No change in initial velocity was observed over a 20-min period. All assays reflected initial velocity conditions with less than 10% of the limiting reactant utilized over the time course of the reaction. The K_m for NADPH approaches the limit of detection for the spectrophotometric assay. As a result, a competitive inhibitor, ATP-ribose, was added at a fixed level to increase the effective K_m. All kinetic parameters were then corrected for the presence of ATP-ribose. The correction term is dependent on the enzyme form represented. As such, no correction for the presence of ATP-ribose is required for values determined at saturating NADPH, i.e., $K_{Ru\ 5\text{-}P}$ and K_{CO_2}. Values determined at nonsaturating NADPH concentrations, i.e., K_{NADPH}, were corrected for the presence of ATP-ribose by dividing by $(1 + I/K_i)$. Product and dead-end inhibition patterns were obtained by varying one reactant at a fixed level of the other reactants (saturating and near K_m) and different fixed levels of the inhibitor. In the direction of oxidative decarboxylation, 6-PG or NADP were fixed at their K_m values when nonsaturating. In the direction of reductive carboxylation, Ru 5-P was maintained at its K_m, but the concentration of CO_2 was maintained at 5 mM (low) and 20 mM (high) instead of K_m and saturating since the K_m for CO_2 is quite high.

Although attainment of the maximum velocity in the direction of oxidative decarboxylation of 6-phosphogluconate presented no problem, it was not possible to attain the maximum rate in the reductive carboxylation direction. As a result, the maximum velocities in both directions were obtained using a common enzyme stock and varying the concentration of one reactant in constant ratio with a second. In the direction of oxidative decarboxylation, data were obtained at pH 7, 100 mM Hepes, and varying the concentration of 6-PG in a constant ratio of 10 with NADP. In the reductive carboxylation direction, data were obtained at pH 7, 100 mM Hepes, with saturating NADPH (200 μM), and varying the concentration of CO_2 in a constant ratio of 0.2 with Ru 5-P. In both cases, extrapolation to infinite concentration of the varied substrate gave the value for V.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots and replots were linear. Data were fitted using the appropriate rate equations and computer programs developed by Cleland (1979). Data conforming to a general bireactant sequential mechanism were fitted using eq 1. Data for competitive and noncompetitive inhibitions were fitted using eqs 2 and 3.

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

$$v = VA/(K_a[1 + I/K_{is}] + A) \quad (2)$$

$$v = VA/(K_a[1 + I/K_{is}] + A[1 + I/K_{ii}]) \quad (3)$$

where v is the initial velocity, V is the maximum velocity, A, B, and I are reactant and inhibitor concentrations, K_a and K_b are the Michaelis constants for A and B, K_{ia} is the dissociation constant for A, and K_{is} and K_{ii} are slope and intercept inhibition constants, respectively. The dissociation constants of substrates from ternary and quaternary complexes are denoted as $K_{i'}$ and $K_{i''}$, respectively.

Attempts to fit the initial velocity data in the direction of reductive carboxylation were fitted using eq. 4 were unsuccessful because the data are not well conditioned. As a result, parameters were estimated graphically.

$$v = VABC/[\text{constant} + (\text{coef } A)A + (\text{coef } B)B + (\text{coef } C)C + K_aBC + K_bAC + K_cAB + ABC] \quad (4)$$

where constant and coefficient terms are mechanism dependent and the product of 3 (constant) or 2 (coef) kinetic parameters. All other terms are as defined above.

Data obtained for the maximum velocities in both reaction directions varying reactants in constant ratios were fitted in double-reciprocal form using

$$y = A + Bx + Cx^2 \quad (5)$$

where y is the value of $1/v$ at any value of the varied reactant, x is the reciprocal of the varied reactant concentration, A is $1/V_{max}$, B and C are coefficients for the initial linear ($x \rightarrow 0$) and exponential ($x \rightarrow \infty$) portions of the curve, respectively.

RESULTS

Initial Velocity Studies in the Absence of Added Inhibitors. Initial velocity patterns were obtained under conditions in which NADP was varied at different fixed levels of 6-PG in the direction of oxidative decarboxylation. The initial velocity pattern obtained intersects to the left of the ordinate, suggesting a sequential kinetic mechanism. In the direction of reductive carboxylation, NADPH was varied at several different fixed levels of Ru 5-P and a single fixed concentration of CO_2. This pattern was then repeated at three different fixed concentrations of CO_2. In the direction of reductive carboxylation, as stated under Materials and Methods, 0.1 mM ATP-ribose was added to each assay to increase the effective K_m for NADPH and ensure a high enough concentration of the reduced dinucleotide to produce linear recorder traces (longer approach to equilibrium) for at least 4 min. In addition, the concentration of CO_2 was always maintained <20 mM. Data from the resulting reciprocal plots were analyzed in terms of the systematic treatment of Viola and Cleland (1982) for a terreactant mechanism. In all cases, initial velocity patterns

Table I: Kinetic Parameters for 6-Phosphogluconate Dehydrogenase from *Candida utilis*[a]

Oxidative Decarboxylation	
$K_{NADP} = 8 \pm 2\ \mu M$	$V/K_{NADP} = (1.6 \pm 0.2) \times 10^5\ M^{-1}\ s^{-1}$
$K_{i\ NADP} = 10 \pm 2\ \mu M$	$V/K_{6\text{-PG}} = (6.1 \pm 0.8) \times 10^4\ M^{-1}\ s^{-1}$
$K_{6\text{-PG}} = 220 \pm 20\ \mu M$	V_{max}[b] $= 11 \pm 1\ s^{-1}$
$K_{i\ 6\text{-PG}} = 280 \pm 80\ \mu M$	
Reductive Carboxylation[c]	
$K_{Ru\ 5\text{-P}} = 1$ mM	coef[Ru 5-P] = 3.2 mM[2]
$K_{NADPH} = 2\ \mu M$	coef[NADPH] = 5.8 mM[2]
$K_{CO_2} = 50$ mM	coef[CO_2] = 0.05 mM[2]
V_{max}[b] $= 0.15 \pm 0.01\ s^{-1}$	constant = 0.25 mM[3]
$V/K_{Ru\ 5\text{-P}} \sim 150\ M^{-1}\ s^{-1}$	
$V/K_{NADPH} \sim 7.5 \times 10^4\ M^{-1}\ s^{-1}$	
$V/K_{CO_2} \sim 3\ M^{-1}\ s^{-1}$	

[a] Data were obtained at pH 7, 100 mM Hepes, and 25 °C as described under Materials and Methods. [b] The maximum velocities were obtained from a variation of the reactants in constant ratio. [c] The data in the direction of reductive carboxylation are not well conditioned to a fit using eq 4. Parameter estimates are from a graphical analysis.

intersect to the left of the ordinate. A list of the kinetic parameters is given in Table I. The maximum rates were obtained by varying reactants in a constant ratio as described under Materials and Methods.

Product Inhibition Studies. In the direction of oxidative decarboxylation, with NADP as the variable substrate, NADPH is a competitive inhibitor whether 6-PG is saturating or nonsaturating, and Ru 5-P is noncompetitive at nonsaturating 6-PG. With 6-PG as the variable substrate, Ru 5-P is a competitive inhibitor whether NADP is saturating or nonsaturating, and NADPH is noncompetitive at nonsaturating NADP. Results from product inhibition studies in the direction of oxidative decarboxylation are given in Table II.

In the direction of reductive carboxylation, with Ru 5-P as the variable substrate, 6-PG is a competitive inhibitor at saturating NADPH at any CO_2 concentration. With CO_2 as the variable substrate, 6-PG is also competitive at saturating NADPH and nonsaturating Ru 5-P. With NADPH as the variable substrate, 6-PG is noncompetitive at nonsaturating Ru 5-P at any CO_2 concentration. Results from product inhibition studies in the direction of reductive carboxylation are given in Table III.

Dead-End Inhibition Studies. ATP-ribose was used as a dead-end analog of both NADP and NADPH. In the direction of oxidative decarboxylation, ATP-ribose is competitive verus NADP at any concentration of 6-PG and noncompetitive with 6-PG. Similarly, 6-SG was used as an analog of 6-PG and is competitive versus 6-PG at any concentration of NADP and is noncompetitive versus NADP at nonsaturating 6-PG. In the direction of reductive carboxylation, ATP-ribose is competitive versus NADPH whether Ru 5-P is saturating or nonsaturating at high or low CO_2 concentrations. 5-Phosphoribonate is competitive versus Ru 5-P at any concentration of NADPH at any CO_2 concentration. With CO_2 as the variable substrate, 5-PRib is competitive at any concentration of NADPH and nonsaturating Ru 5-P (Table III). 5-PRib is noncompetitive versus NADPH at any concentration of CO_2 and nonsaturating Ru 5-P. A summary of the data obtained from dead-end inhibition studies in the direction of oxidative decarboxylation and reductive carboxylation is given in Tables II and III, respectively.

DISCUSSION

Initial Velocity Studies in the Absence of Added Inhibitors. In the direction of oxidative decarboxylation of 6-phosphogluconate, the initial velocity pattern obtained by varying NADP at different fixed levels of 6-PG intersects to the left of the ordinate, suggesting a sequential kinetic mechanism. In the opposite reaction direction, a systematic study was carried out in which NADPH was varied at several different fixed levels of Ru 5-P and a single fixed concentration of CO_2, and this pattern was repeated at several different concentrations of CO_2. A fit of these data to the equation for a random terreactant mechanism indicates that all denominator terms are present consistent with a random mechanism that at least approximates rapid equilibrium. The ratio of the maximum velocities in the directions of oxidative decarboxylation and reductive carboxylation is approximately 75. In addition, although the oxidized and reduced dinucleotide substrates, as well as 6-PG and Ru 5-P, have K_m values within a factor of 4 of one another, the large K_m value for CO_2 suggests very little role for this enzyme in the CO_2 fixation direction.

Product Inhibition. The competitive product inhibition exhibited by both products vs the reactant (NADPH vs NADP, Ru 5-P vs 6-PG, and 6-PG vs Ru 5-P) to which they are analogs at nonsaturating concentrations of the fixed substrate suggests a rapid equilibrium mechanism. That the competitive pattern persists even under conditions of saturation by the fixed substrate is additionally indicative of dead-end E:NADP:Ru 5-P and E:NADPH:6-PG complexes. Consistent with this interpretation, product inhibition patterns for NADPH vs 6-PG, Ru 5-P vs NADP, and 6-PG vs NADPH at nonsaturating NADP, 6-PG, and Ru 5-P, respectively, are noncompetitive, indicative of combination of product to both free enzyme and the binary complex of enzyme and the variable substrate. For this kind of mechanism, the products behave as dead-end analogs of the variable substrates. Thus, the K_{is} obtained for NADPH vs NADP at saturating 6-PG should be identical to the K_{ii} obtained for NADPH vs 6-PG once corrected for the presence of NADP. The expression relating the two is app$K_i = K_i\ (1 + NADP/K_{NADP})$. With NADP equal to K_{NADP}, the K_i is half the calculated K_i given in Table II. Thus values of 4.2 μM and 2.4 μM for E:6-PG:NADPH and 310 and 210 μM for E:NADP:Ru 5-P can be compared. Considering the low values of the K_i for NADPH and standard errors, these values are in reasonable agreement. The values of K_{is} obtained at nonsaturating concentrations of the fixed substrate are also not true K_i values and must be corrected for the concentration of the fixed substrate. The correction term is much more complex in this case because the inhibitor has a choice of binding to either E or EB (if it is an analog of A such as NADPH) and the affinities need not be identical. The apparent K_{is} in this case is equal to $[(K_{is}K_{ii}K_b)/(K_{is}K_bK_{ii} + K_{is}K_aB)][(K_{ia}K_b + K_aB)/B]$, but with B equal to K_b, this reduces to $[(K_{is}K_{ii})/(K_{ia}K_{ii} + K_{is}K_a)][K_{ia} + K_b]$. Using values of 8, 10, and 4.2 μM, for K_a, K_{ia}, and K_{ii}, respectively, a value of 3 μM is calculated for E:NADPH. A second value for this constant can be calculated using the apparent K_{is} obtained from the noncompetitive pattern. In this case, the correction is for the presence of NADP and is $K_{is}\ (1 + A/K_{ia})$. Applying this correction gives a value of 1.8 μM for K_{is}. Since true values of the dissociation constant for NADPH from E:NADPH:6-PG and E:NADPH are equal, it thus appears that the presence of one of the reactants does not affect the affinity of the product. The competitive pattern for 6-PG vs CO_2 at saturating NADPH and nonsaturating Ru 5-P is also indicative of combination of 6-PG to the E:NADPH complex and also tends to indicate that CO_2 or HCO_3^- does have a weak binding site. In agreement with this, the K_i for 6-PG obtained from product inhibition vs Ru 5-P increases (77 μM) when CO_2 is

Table II: Product and Dead-End Inhibition Patterns for 6-Phosphogluconate Dehydrogenase from *Candida utilis* in the Direction of Oxidative Decarboxylation[a]

varied substrate	fixed substrates	inhibitor	pattern	K_{is} ± SE (μM)	K_{ii} ± SE (μM)	enzyme form[c]
NADP	6-PG (ns)[b]	NADPH	C	3.5 ● 0.5 (3)		E, E:NADP
NADP	6-PG (s)	NADPH	C	2.3 ± 0.4		E:6-PG
6-PG	NADP (ns)	NADPH	NC	3.2 ± 0.8 (1.8)	8.3 ● 0.2 (4.2)	E, E:6-PG
6-PG	NADP (ns)	Ru 5-P	C	430 ± 80 (270)		E, E:6-PG
6-PG	NADP (s)	Ru 5-P	C	210 ± 40		E:NADP
NADP	6-PG (ns)	Ru 5-P	NC	130 ± 3 (80)	620 ± 140 (310)	E, E:NADP
NADP	6-PG (ns)	ATP-R[d]	C	6.4 ± 0.8 (4.2)		E, E:NADP
NADP	6-PG (s)	ATP-R	C	12 ± 2		E:6-PG
6-PG	NADP (ns)	ATP-R	NC	8.5 ± 0.1 (4.7)	33 ± 5 (16)	E, E:6-PG
6-PG	NADP (ns)	6-SG	C	1600 ± 200 (1000)		E, E:6-PG
6-PG	NADP (s)	6-SG	C	6100 ± 1100		E:NADP
NADP	6-PG (ns)	6-SG	NC	1200 ± 200 (700)	13500 ± 3200 (6750)	E, E:NADP

[a] Data were attained at pH 7, 100 mM Hepes, and 25 °C. [b] The abbreviations (ns) and (s) indicate the fixed substrate was maintained equal to its K_m or >10 times its K_m, respectively. C and NC represent competitive and noncompetitive inhibition, respectively. Values in parentheses represent those values corrected for the presence of the fixed substrate. [c] Enzyme form represents the enzyme form to which inhibitor binds. The first enzyme form listed refers to the predominate form present where the slope inhibition constant is considered, while the second reflects the intercept inhibition constant. [d] ATP-R is ATP-ribose.

Table III: Product and Dead-End Inhibition Patterns for 6-Phosphogluconate Dehydrogenase from *Candida utilis* in the Direction of Reductive Carboxylation[a]

varied substrate	fixed substrates	inhibitor	pattern	K_{is} ± SE (μM)	K_{ii} ± SE (μM)
Ru 5-P	NADPH (s)[b] CO_2 (high)	6-PG	C	77 ± 16	
Ru 5-P	NADPH (s) CO_2 (low)	6-PG	C	7 ± 1	
NADPH	Ru 5-P (ns) CO_2 (high)	6-PG	NC	320 ± 200	1130 ± 700
NADPH	Ru 5-P (ns) CO_2 (low)	6-PG	NC	150 ± 60	600 ± 200
CO_2	Ru 5-P (ns) NADPH (s)	6-PG	C	330 ± 40	
NADPH	Ru 5-P (s) CO_2 (high)	ATP-R[c]	C	46 ± 21	
NADPH	Ru 5-P (s) CO_2 (low)	ATP-R	C	100 ± 40	
NADPH	Ru 5-P (ns) CO_2 (high)	ATP-R	C	21 ± 5	
NADPH	Ru 5-P (ns) CO_2 (low)	ATP-R	C	24 ± 6	
Ru 5-P	NADPH (ns) CO_2 (high)	ATP-R	NC	28 ± 15	90 ± 30
Ru 5-P	NADPH (ns) CO_2 (low)	ATP-R	NC	17 ± 6	270 ± 80
CO_2	NADPH (ns) Ru 5-P (s)	ATP-R	NC	90 ± 40	66 ± 40
CO_2	NADPH (ns) Ru 5-P (ns)	ATP-R	NC	53 ± 12	50 ± 20
Ru 5-P	NADPH (s) CO_2 (high)	5-PRib	C	4900 ± 1200	
Ru 5-P	NADPH (s) CO_2 (low)	5-PRib	C	3500 ± 800	
Ru 5-P	NADPH (ns) CO_2 (high)	5-PRib	C	760 ± 220	
Ru 5-P	NADPH (ns) CO_2 (low)	5-PRib	C	190 ± 50	
NADPH	Ru 5-P (ns) CO_2 (high)	5-PRib	NC	3700 ± 1500	28900 ± 2200
NADPH	Ru 5-P (ns) CO_2 (low)	5-PRib	NC	1100 ± 300	4700 ± 700
CO_2	Ru 5-P (ns) NADPH (s)	5-PRib	C	24600 ± 6000	
CO_2	Ru 5-P (ns) NADPH (ns)	5-PRib	C	900 ± 100	

[a] Data were obtained at pH 7, 100 mM Hepes, and 25 °C. [b] The abbreviations (ns) and (s) indicate the fixed substrate was maintained equal to its K_m or >10 times its K_m, respectively. High and low represent CO_2 concentrations of 20 and 5 mM, respectively. C and NC represent competitive and noncompetitive inhibition, respectively. [c] ATP-R is ATP-ribose.

increased to 20 mM compared to the value (7 μM) measured at 5 mM. Unfortunately, corrections for K_i values cannot be applied in the reductive carboxylation direction because of the inability to saturate with CO_2.

Dead-End Inhibition. The dead-end patterns obtained are qualitatively identical to the product inhibition patterns obtained; that is, ATP-ribose is competitive vs NADP at any level of 6-PG and noncompetitive vs 6-PG while 6-SG is competitive vs 6-PG at any level of NADP and noncompetitive vs NADP. Moreover, the true K_i values can again be calculated using the same expression as those used for product inhibition. The K_{is} obtained for ATP-ribose vs NADP at saturating 6-PG is 12 μM, and the K_{ii} obtained for ATP-ribose vs 6-PG is 16 μM when corrected for the presence of NADP. In the case of 6-SG, the K_{is} obtained for 6-SG vs 6-PG at saturating NADP is 6.1 mM, and the K_{ii} obtained for 6-SG vs NADP is 6.7 mM when corrected for the presence of 6-PG. Thus, values of 12 and 16 μM for E:6-PG:ATP-Ribose and 6.1 and 6.7 mM for E:NADP:6-SG can be compared, and when standard errors are taken into consideration, these values are in excellent agreement. Likewise, values for E:ATP-ribose can be calculated as for E:NADP above. Thus, true values of 4.2 and 4.7 μM are calculated from the measured values of 6.4 and 8.5 μM for E:ATP-ribose, respectively, while true values of 1000 and 700 μM are calculated from measured values of 1600 and 1200 μM, respectively, for E:6-SG. All of these values are in excellent agreement and argue for the rapid equilibrium random mechanism. Unlike the case with the products of the reaction, the presence of 6-PG decreases the affinity for ATP-ribose by about 3-fold while the presence of NADP decreases the affinity for 6-SG by about 6-fold. These data suggest some differences in the binding of products compared to dead-end inhibitors.

In the direction of reductive carboxylation, ATP-ribose is competitive vs NADPH irrespective of the concentrations of Ru 5-P and CO_2 and noncompetitive vs Ru 5-P and CO_2 whatever the concentration of CO_2 and Ru 5-P, respectively. In the case of ATP-ribose, the K_{is} obtained for ATP-ribose vs NADPH at saturating Ru 5-P and high CO_2 is 46 μM, and the K_{ii} obtained for ATP-ribose is 45 μM when corrected for NADPH. These values obtained for the E:Ru 5-P:CO_2 complex are also in excellent agreement. The inhibition exhibited by 5-PRib is qualitatively similar to that exhibited by ATP-ribose in that it is competitive vs Ru 5-P irrespective

of the concentrations of NADPH and CO_2 and noncompetitive vs NADPH irrespective of the CO_2 concentration. As stated above for product inhibition, correction of the apparent K_i values cannot be made in the reverse direction. In the case of the 5-PRib vs Ru 5-P, however, we see a consistent decrease in K_{is} as the concentrations of NADPH and/or CO_2 decrease. These data are in agreement with one of two possibilities: (1) CO_2 has a binding site, and this interferes with the binding of 5-PRib or 6-PG or, more likely, (2) HCO_3^- binds to the phosphate site, and this interferes with binding of the smaller 5-PRib as well as 6-PG.

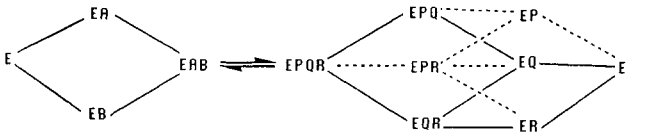
Assignment of a rapid equilibrium random mechanism to the *Candida utilis* 6-PGDH allows assignment of the constant and coefficient terms of Table I after derivation of the rate equation in the direction of reductive carboxylation using the method of Cha (1968). The rate equation follows:

$$v = VPQR/(K_{ir}K_{iq'}K_p + K_{iq'}K_pR + K_{ir'}K_pQ + K_{ir'}K_qP + K_pQR + K_qPR + K_rPQ + PQR) \quad (6)$$

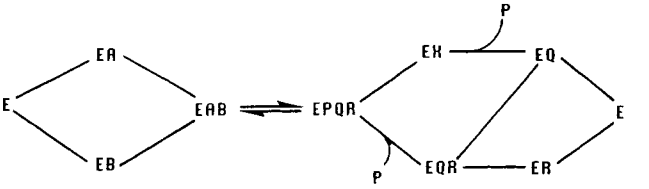
where P, Q, and R are the concentrations of CO_2, Ru 5-P, and NADPH, respectively, K_p, K_q, and K_r are the Michaelis constants for CO_2, Ru 5-P, and NADPH, respectively, $K_{iq'}$ is the dissociation constant for Ru 5-P from the E:NADPH:Ru 5-P ternary complex, and $K_{ir'}$ is the dissociation constant for NADPH from either the E:CO_2:NADPH or E:Ru 5-P:NADPH ternary complexes. The latter need not be the same, but in the E:NADPH and E:NADPH:6-PG complexes they are identical.

Comparison of the kinetic mechanism of 6-phosphogluconate dehydrogenase from *Candida utilis* and that of the NAD-malic enzyme from *Ascaris suum* yields interesting similarities and differences. As stated before, both enzymes catalyze similar reactions in that both yield a ketone, CO_2, and a reduced dinucleotide as products. However, malic enzyme is reported to have a pseudo-Ter-Quad random kinetic mechanism in which Mg^{2+} acts as a pseudoreactant (Mallick et al., 1991) while 6-PGDH has a Bi-Ter random kinetic mechanism with no divalent metal ion required for activity. Although both enzymes have random mechanisms, malic enzyme has a steady-state mechanism in which catalysis is not the only slow step (Mallick et al., 1991) while 6-PGDH has a rapid equilibrium mechanism in which the pathway that contains the catalytic step(s), i.e., interconversion of the central complexes is slow for the overall reaction or a steady-state random mechanism that closely approximates rapid equilibrium. There is no evidence from the data presented with NADP as the dinucleotide substrate for a slow conformational change in the 6-PGDH reaction as is the case with the E:NAD complex for malic enzyme (unpublished data of R. Rajapaksa in this lab). However, data presented using NAD as an alternative substrate are consistent with a conformational change by dinucleotide binding (Berdis and Cook, unpublished results). Both enzymes have dead-end product complexes of the type E:NAD(P):ketone and E:NAD(P)H:alcohol, but, with malic enzyme, the metal ion must additionally be bound prior to the addition of malate or pyruvate. If the metal ion is not present, dead-end substrate or product complexes such as E:NAD:malate and E:NADH:pyruvate are observed. Neither malic enzyme (Mallick et al., 1991) nor 6-PGDH appear to have a binding site for CO_2 (although this has not been conclusively ruled out for 6-PGDH). As a result, it is likely that CO_2 reacts directly with an enzyme:enol intermediate for both enzymes (enol of oxalacetate for malic enzyme and that of 3-keto-6-phosphogluconate for 6-PGDH).

Scheme I



Scheme II



Conclusions. The mechanism for 6-phosphogluconate dehydrogenase can be described by Scheme I, where A, B, P, Q, and R represent NADP, 6-PG, CO_2, Ru 5-P, and NADPH, respectively. The EAQ and EBR complexes are present in both reaction directions since the mechanism is random. The solid lines reflect reversible steps while the dotted lines indicate that there may be no binding site for CO_2 and the latter may react directly as shown in Scheme II, where all terms are as defined above and X is 3-keto-6-phosphogluconate. The latter is generated by direct attack of the C-1 carbanion of Ru 5-P on CO_2. Which of these mechanisms is correct must await further study.

REFERENCES

Anderson, B. M., Anderson, C. D., & Yoon, H. (1989) *Biochim. Biophys. Acta 994*, 75.
Bellini, T., Dallocchio, F., & Matteuzzi, M. (1985) *Biochem. J. 227*, 305.
Cha, S. (1968) *J. Biol. Chem. 243*, 820.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Cook, P. F., & Cleland W. W. (1981) *Biochemistry 21*, 1797.
Dalziel, K., & Topham, C. M. (1986) *Eur. J. Biochem. 156*, 555.
Hansen, W. H., D'Orazio, R. E., & Dyson, J. E. D. (1973) *Arch. Biochem. Biophys. 154*, 623.
Holten, D., & Procsal, D. (1972) *Biochemistry 11*, 1310.
Horecker, B. L. (1957) *Methods Enzymol. 3*, 172.
Mallick, S. M., Harris, B. G., & Cook, P. F. (1991) *J. Biol. Chem. 266*, 2732.
Pontremoli S., de Flora, A., Grazi, E., Mangiarotti, G., Bonsignore, A., & Horecker, B. L. (1961) *J. Biol. Chem. 236*, 2975.
Rendina, A. R., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 6257.
Rutter, W. J., & Lardy, H. A. (1958) *J. Biol. Chem. 233*, 374.
Siebert, G., Carsiotis, M., & Plaut, G. W. E. (1957) *J. Biol. Chem. 226*, 977.
Villet R. H., & Dalziel K. (1972) *Eur. J. Biochem. 27*, 244.
Viola, R. E., & Cleland, W. W. (1982) *Methods Enzymol. 87*, 353.